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Astrocytes mediate bone marrow stromal cell transplantation enhanced glial cell derived neurotrophic factor (GDNF) production in the ischemic boundary zone after stroke in adult rats

LH Shen^a, Y Li^a, and M Chopp^{a,b}

^a Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI, USA

^b Department of Physics, Oakland University, Rochester, MI, U.S.A

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF), a protein of the transforming growth factor- β (TGF- β) superfamily, was initially cloned and purified from the B49 glial cell line ¹. As a potent neurotrophic factor, GDNF promotes the survival of central dopaminergic ^{1–3}, noradrenergic ⁴ as well as motor neurons ^{5, 6}. Moreover, GDNF enhances striatal neurogenesis after stroke ^{7–9}. Efforts have been made to deliver exogenous GDNF directly into the brain ^{10–12}, by adenoviral vector ¹³, or by gene-modified mesenchymal stem cells ¹⁴ in different models of cerebral ischemia, and the efficacy of GDNF treatment of experimental stroke has been well established. Animals receiving GDNF have smaller infarct volume, less severe cerebral edema, and a reduced number of apoptotic cells in the penumbral zone ^{14–16}. GDNF expression is low in normal adult rat brain, but can be up-regulated by a variety of central nervous system (CNS) insults including focal brain ischemia ^{17–20}. Taken together, these data suggest that manipulation of endogenous GDNF levels may facilitate the self-repair efforts of the brain against ischemic attack, and therefore, could be a new target in the treatment of stroke.

Intravenous transplantation of bone marrow stromal cells (BMSCs) ameliorates functional deficits in rodent cerebral ischemia models ^{21–23} as well as in stroke patients ²⁴. BMSCs are a heterogeneous subpopulation of bone marrow cells including mesenchymal stem and progenitor cells. Although these cells can release a variety of neurotrophic factors in response to ischemic environment ^{25–27}, the limited number of BMSCs in the brain raises doubt about the importance and weight of factors secreted by BMSC per se in the process of functional recovery. Being the most abundant cells in the brain, astrocytes provide many supportive activities essential for neuronal function under physiological circumstances ^{28, 29}. After various insults, local astrocytes undergo cellular hypertrophy, hyperplasia and become “reactive”. Reactive astrocytes promote brain plasticity and recovery from stroke, and the beneficial effects of reactive astrocytes are enhanced in the ischemic brain after BMSC transplantation ^{30–32}. In the present study, we hypothesize that BMSCs in the penumbral zone activate local astrocytes, the latter release large amount of GDNF, which contributes to the functional improvement.

MATERIALS AND METHODS

Experiments were performed on adult male Wistar rats (2–3 months old). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal middle cerebral artery occlusion (MCAo) model

Transient MCAo was induced using a method of intraluminal vascular occlusion modified in our laboratory³³. Briefly, rats (n=21) were initially anesthetized with 3.5% isoflurane and maintained with 1.0–2.0% isoflurane in 70% N₂O and 30% O₂ using a face mask. The rectal temperature was controlled at 37°C with a feedback regulated water heating system. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed. A length of 4.0 monofilament nylon suture (18.5–19.5 mm), determined by the animal weight, with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. Two hours after MCAo, animals were reanesthetized with isoflurane, and reperfusion was performed by withdrawal of the suture until the tip cleared the lumen of the ECA. The following day, the modified neurologic severity score (mNSS) test was employed to evaluate the neurological functions of the animals, and only animals showing obvious deficits were selected for the subsequent studies.

Cell transplantation procedures

Primary cultures of bone marrow stromal cells were obtained from donor young adult male rats and BMSCs were separated, as previously described²⁷. At 24 hrs post-ischemia, randomly selected rats received BMSCs administration. Animals were anesthetized with 3.5% isoflurane and then maintained with 1.0–2.0% isoflurane in N₂O:O₂ (2:1). Approximately 3×10^6 BMSCs in 1 ml phosphate buffered saline (PBS, n=11) or control fluid (1 ml PBS, n=10) were slowly injected over a 5-min period into each rat via the tail vein. All animals were euthanized at 7 days after treatment, among which, 6 brains (n=3/group) were employed for laser capture purpose, and the other 15 (7 from the PBS group, 8 from the BMSC treatment group) were processed for immunostaining.

Immunohistochemical assessment

For paraffin embedded brain tissues, a series of 6- μ m-thick slides were cut from a standard paraffin block obtained from the center of the lesion of the forebrain, corresponding to coronal coordinates for bregma ~ -1 – -1 mm³⁴ for immunostaining. After deparaffinizing, brain sections were placed in boiled citrate buffer (pH 6) in a microwave oven (650 to 720 W). Following blocking in normal serum, sections were incubated with antibodies against GDNF (dilution, 1:50, Santa Cruz, Santa Cruz, CA), Ki67 (dilution, 1:300, lab vision, Fremont, CA) or doublecortin (DCX, 1:250, Santa Cruz, Santa Cruz, CA) at 4°C overnight. Then the sections were incubated with avidin-biotin-horseradish peroxidase complex and developed in 3'3' diaminobenzidine tetrahydrochloride (DAB, for neurocan). Double immunohistochemical staining was employed to detect the cellular co-localization of GDNF with astrocyte markers, glial fibrillary acidic protein (GFAP, dilution, 1:10,000, Dako, Carpinteria, CA); neuronal marker, microtubule-associated protein 2 (MAP2; dilution 1:200, Chemicon, Temecula, CA); oligodendrocyte marker, CNPase (dilution, 1:100, Chemicon, Temecula, CA); and endothelial cell marker, von Willebrand factor (vWF; dilution 1:400, Dako, Carpinteria, CA). The fluorescein isothiocyanate conjugated antibody (FITC, Jackson Immunoresearch, West Grove, PA) and CY3 conjugated antibody (Jackson Immunoresearch, West Grove, PA) were employed for double immunoreactivity identification. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that the primary antibodies were omitted.

The terminal deoxynucleotidyl transferase (TdT) –mediated dUTP-biotin nick end labeling (TUNEL) method (in situ Apoptosis Detection Kit, Chemicon, Billerica, MA) was used to assess in situ apoptotic detection^{1,5}. The TUNEL method is based on the specific binding of TdT to 3'-OH ends of DNA and the ensuing synthesis of polydeoxynucleotide polymer

cells. The staining was performed according to the procedures provided by the manufacturer.

Laser capture microdissection (LCM) of reactive astrocytes

Brain coronal sections (8 μ m) were cut on a cryostat set at -20°C and kept at -80°C until processing. For the immunostaining, the sections localized to the territory supplied by the MCA were air-dried for 30 secs and fixed in freshly prepared 4°C cold acetone for 2 mins. The coronal sections were incubated with GFAP antibody at 1:50 dilution for 5 mins, and then incubated with CY3-conjugated F(ab')₂ anti-rabbit IgG secondary antibody for 5 mins. After air-drying for 5 mins, GFAP positive reactive astrocytes along the ischemic boundary were cut using a Leica LMD6000 system. Approximately 1,000 cells were dissected and collected in Eppendorf tubes containing 100 μ l of lysis buffer. The samples were stored in -80°C before RNA isolation.

Cell culture

Rat cortical astrocytes, phenotype 1, were obtained from the American Type Culture Collection (ATCC). Cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) with 10% FBS (Gibco), containing penicillin–streptomycin on 75 cm² tissue culture flasks (Corning) in 37°C , 5% CO₂.

Astrocyte and BMSC coculture

8×10^5 astrocytes per well were cultured in 6-well plates. In the same plate, 4×10^5 BMSCs (BMSC to astrocyte ratio, 1:2), were cocultured in the upper chamber of transwell cell inserts. Thus, astrocytes and BMSCs shared the same medium environment, yet were not in direct contact.

Oxygen-glucose deprivation (OGD) and reoxygenation

As previously described³², OGD was induced with an anaerobic chamber (model 1025, Forma Scientific, OH). The 10% FBS, high glucose DMEM was replaced with 1 ml of glucose and serum-free DMEM in the astrocyte culture. Then the astrocyte culture was transferred to the anaerobic chamber saturated with 85% N₂/10% H₂/5% CO₂, at 37°C . Astrocytes were incubated in this OGD condition for 5 hrs. Then astrocyte cultures were removed from the anaerobic chamber, rinsed with PBS, and fed with 3 ml fresh half glucose and 5% FBS DMEM. BMSCs were then introduced to the astrocyte culture and were incubated together with astrocytes for an additional 4 hrs under reoxygenation conditions.

Real-time reverse transcriptase-polymerase chain reaction (real time RT-PCR)

Quantitative polymerase RT-PCR was performed using SYBR Green system. Total RNA from LCM cells or cultured astrocytes was extracted using Absolutely RNA nanoprep and miniprep kit, respectively (Stratagene, La Jolla, CA). RNA was subsequently reverse transcribed to cDNA with SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed afterwards. Primer (Invitrogen) concentrations (10nM) were optimized before use. A 1x SYBR Green PCR master kit was used with the appropriate concentrations (10nM) of forward and reverse primers in a total volume of 20 μ l. PCR reactions contained 1 μ l cDNA. Optimization was performed of each gene-specific primer prior to the experiment to confirm that 10nM primer concentrations did not produce nonspecific primer-dimer amplification signal in no-template control wells. Quantitative RT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) by using 3-stage program parameters provided by the manufacturer as follows; 2 mins at 50°C , 10 mins at 95°C , and then 40 cycles of 15 s at 95°C and 1 min at 60°C . Specificity of the produced amplification product was confirmed by examination of dissociation

reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during RT-PCR. RT-PCR products were run on 2% agarose gels to confirm that correct molecular sizes were present. Each sample was tested in triplicate with quantitative RT-PCR, and samples obtained from 3 rats each group (for LCM) or three independent experiments were used for analysis of relative gene expression data using the $2^{-\Delta\Delta CT}$ method. Primers employed were: β -actin: 5'-CCA TCA TGA AGT, GTG ACG TTG-3' (fwd), 5'-CAA TGA TCT TGA TCT TCA TGG TG-3' (rev). GDNF: 5'-GCC GAG ACA ATG TAC GAC AA-3' (fwd), 5'-CTG GAG CCA GGG TCA GAT AC-3' (rev).

Quantification and statistical analysis

Immunoreactive cells were analyzed with NIH image software (Image J) based on the evaluation of an average of three histology slides (6 μ m thick, 54 μ m interval, every 10th slide) from the standard block of each animal. Ki-67-positive proliferating cells in the SVZ were counted in a 100-mm-thick band encompassing the ependymal layer of the lateral ventricles on each slide. For DCX, all the immunopositive areas along the subventricular zone (SVZ) and the ischemic boundary zone (IBZ) were digitalized and summed in each slide. Eight fields of view along the IBZ were digitalized under a 40 $\times$ objective, the number of TUNEL positive cells was summed for apoptotic assay, and the percentage of immunoreactive area was calculated and averaged for GDNF staining. The gene expression fold change data were tested between normoxic, OGD, and OGD + BMSCs conditions by one-way ANOVA. Student's t-test was used to analyze all the in vivo related immunohistochemical and LCM data between 2 groups. All data are presented as mean $\pm$ SE. A value of $P < 0.05$ was taken as statistically significant.

RESULTS

Immunochemical changes in the IBZ and SVZ

Immunohistochemistry of the contralateral brain of the MCAo rats for GDNF showed little positive signal (data not shown). In contrast, a substantial increase in GDNF expression was observed in the peri-infarct tissue of the cerebral cortex as well as striatum 8 days after ischemic attack (Fig 1A). Due to the overlap of GDNF positive signal and GFAP positive reactive astrocyte distribution around the injury sites, double fluorescent immunostaining was employed to discern the spatial relationship of these 2 proteins. As shown in Fig 1D–F, GDNF signals aggregated around and within the cytoplasm of GFAP expressing reactive astrocytes, which indicates that reactive astrocytes in the IBZ synthesize GDNF. In addition, double staining of GDNF with neuronal marker MAP2, mature oligodendrocyte marker CNPase, or endothelial marker vWF shows little, if any co localization (data not shown), which supports our hypothesis that astrocytes are the major source of GDNF at 8 days after ischemic attack. Quantitative analysis shows that animals receiving BMSC transplant have significantly higher level of GDNF compared to control MCAo alone rats (Fig 1A–C).

TUNEL staining identifies cells undergoing apoptosis. Within the reference coronal 6 μ m thick section, the number of apoptotic cells measured was reduced in the IBZ in BMSC treated rats compared with rats 8 days after MCAo alone (76 ± 13.4 vs. 145 ± 25.7 , $P < 0.05$, Fig 1G–J). To test whether increased GDNF expression by BMSC treatment after stroke negatively correlates with the number of apoptotic cells in IBZ, correlation analyses of GDNF with the number of apoptotic cells was performed. GDNF expression significantly correlated with the decrease of the number of apoptotic cells ($r = 0.71$, $P < 0.05$).

The Ki-67 protein, which is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), is a marker of cell proliferation³⁵. As shown in Fig 2A–C, the majority of Ki-67 immunoreactive cells were located along the

SVZ. BMSC treatment also significantly increased the number of proliferating cells in the SVZ compared with control values ($P < 0.05$).

DCX is a microtubule-associated protein expressed almost exclusively in immature neurons³⁶. It has been widely used as a marker for migrating neuroblasts as well as an indicator for neurogenesis^{36, 37}. Our data indicated that BMSC treatment significantly increased DCX density compared to the MCAo alone group ($P < 0.05$, Fig 2D–F).

Effects of BMSCs on GDNF expression in reactive astrocytes (in vivo & in vitro)

LCM permits extraction of single cells from specific microscopic regions of tissue sections. Combined with GFAP immunostaining, this technique allows the isolation of individual astrocytes within the brain tissue (Fig 3A–D). To identify the source of GDNF, and to investigate whether or not BMSCs conferred alteration of this neurotrophic factor resulted from the regulation of gene expression, we dissected 1,000 reactive astrocytes along the IBZ from each animal and applied RT-PCR. Our data show that GDNF gene expression level was almost five times higher in astrocytes isolated from BMSC treated brain, compared to that of MCAo alone control brain (4.9 ± 0.97 vs 1 ± 0.23 , $n=3/\text{group}$, $P < 0.05$). These data share the same trend with alteration of protein expression detected by semi-quantitative immunostaining analysis, which suggests that the change of GDNF protein expression is initiated by alterations at the gene level.

To obtain further evidence for the astrocytic origin of GDNF, and to investigate the impact of hypoxic condition as well as BMSC coculture on GDNF gene expression in astrocytes, rat astrocytes were subjected to 5 hrs of OGD and 4 hrs of reoxygenation, and RT-PCR was performed. In agreement with our in vivo data (Fig 3E), GDNF gene expression was significantly up-regulated in the OGD group compared to astrocytes in the normoxic control group (2.43 ± 0.35 fold of normoxia, $n = 3$, $P < 0.05$); the presence of BMSC during reoxygenation further strengthened this increment to 4.76 ± 0.85 fold of normoxia ($n = 3$, $P < 0.01$ vs normoxia; $P < 0.05$ vs OGD).

DISCUSSION

Our data demonstrate that the GDNF level is significantly increased in the peri-infarct area at 8 days after MCAo. Reactive astrocytes are a source for GDNF. Intravenous BMSC transplantation at 24 hrs after ischemia significantly increases GDNF expression, decreases the number of apoptotic cells, and GDNF level is negatively related to the number of apoptotic cells in the IBZ. Moreover, BMSC treatment facilitates the proliferation and migration of neuroblasts. Consistent with the above findings, in vitro cell culture data show that GDNF gene expression in astrocytes is up-regulated after OGD, and BMSC coculture during reoxygenation further and significantly increases this change.

BMSCs are a mixed cell population including stem as well as progenitor cells. When systemically administered, these cells migrate toward sites of ischemic brain tissue, and more than 80% of MSCs that reach the brain are localized to the ipsilateral hemisphere, the majority of which congregate in the ischemic border zone^{25, 38}. BMSCs in the IBZ respond to the ischemic brain environment, act like small molecular factories, and stimulate the production of a set of neurotrophins and growth factors, such as brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), and nerve growth factor (NGF), etc^{26, 39–43}. These factors in concert may promote and facilitate the functional recovery. As only a very small portion (<5%) of injected BMSCs can be detected in brain parenchyma at 14 days after transplantation⁴⁴, it is unlikely that bioactive factors released by local BMSCs per se are the only and direct contributors for the improvement of neurological deficits. Our previous studies indicate that BMSCs rescue partially damaged astrocytes and induce

astrocytic production of an array of trophic factors like BDNF, bFGF, NGF, and BMP2/4 etc^{26, 30, 31, 43, 45, 46}. Taking into account the close spatial relationship of BMSCs and numerous reactive astrocytes in the IBZ, we propose that local reactive astrocytes act as the mediators between the limited number of BMSCs in the brain and the robust functional recovery by releasing neurotrophic factor, e.g. GDNF. In agreement with this hypothesis, our in vivo data show: 1) GDNF immunostaining localizes around and within reactive astrocytes in the IBZ; 2) GDNF protein is significantly increased in BMSC treated brain; and 3) GDNF gene expression level is markedly increased in astrocytes collected from BMSC treated brain. In addition, the in vitro data confirm that: 1) GDNF gene expression in astrocytes is up-regulated after OGD; and 2) BMSC coculture during reoxygenation further increases GDNF gene expression level.

As a method of isolating pure cells of interest from specific microscopic regions of tissue sections, LCM allows the detailed analysis of gene expression profile of populations of homogeneous brain cells, and therefore, provides valuable information on the pathological processes. In the present study, GFAP positive reactive astrocytes were collected for gene analysis, and the RNA data from pure astrocytes provide an extremely clear view of the effect of BMSCs on local astrocytes under ischemic conditions. In addition, from Fig 3C, we note that astrocytes adjacent to brain damage are activated by focal ischemia.

GDNF belongs to a family of related proteins, which include GDNF, neurturin, artemin and persephin. Four different receptors, GDNF receptor $\alpha 1-4$ (GFR $\alpha 1-4$), have been identified to interact with these ligands, respectively. The binding of GDNF to GFR $\alpha 1$ primarily activates transmembrane tyrosine kinase c-Ret and induces further downstream signaling⁴⁷. GDNF expression is upregulated after transient brain ischemia^{20, 48}, and so are GDNF receptor proteins, GFR $\alpha 1$ and c-Ret^{15, 47}. Considering the well-established neuroprotective effect of GDNF, the above data suggest that GDNF molecules are endogenous neuroprotective agents that can be activated during ischemia. Previous studies demonstrate that the upregulation of GDNF persists at least to 7 days after ischemic attack, and reactive astrocytes are the major sources of this neurotrophic factor⁴⁸. Consistent with the above observations, our data show that 1) the majority of GDNF positive signal is colocalized with the astroglial marker GFAP; 2) BMSC treatment significantly increases GDNF level at 8 days after ischemia, and 3) GDNF level is negatively correlated with the number of apoptotic cells in the IBZ. In addition to the neuroprotective role, GDNF also increases striatal neurogenesis after stroke¹¹. Our data indicate that BMSC-treated rats have increased proliferation and migration of neuroblasts in the SVZ compared to control animals, possibly attribute to the fact that GDNF is a very diffusible protein⁴⁹ and it may readily diffuse to the SVZ.

In summary, we demonstrate that BMSC transplantation significantly increases GDNF expression, enhances the proliferation and migration of neuroblasts from the SVZ, and decreases the number of apoptotic cells in the IBZ at 8 days after MCAo. Modulation of GDNF released from local reactive astrocytes may, at least, partially explain the anti-apoptotic effect of BMSCs in the IBZ as well as their neurogenesis facilitating effect in the SVZ.

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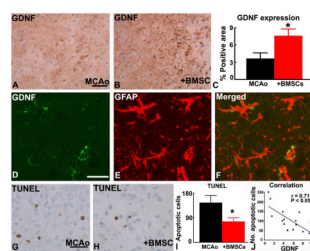


Fig 1. BMSC treatment significantly increases GDNF expression (A–C) and decreases the number of apoptotic cells in the ischemic boundary zone (G–I). The level of GDNF is negatively related to the number of apoptotic cells (J). Double staining shows that GDNF protein is localized within or near GFAP positive astrocytes (D–F). * $P < 0.05$ vs MCAo alone. Scale bars, A, B = 50 μm , D–F = 25 μm , G, H = 25 μm .

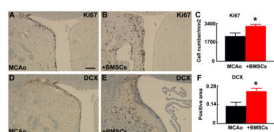


Fig 2. BMSC significantly increases the number of proliferating Ki-67 positive cells in the subventricular zone (A–C); There are more DCX positive neuroblasts in the BMSC group compared to the MCAo alone group (D–F). * $P < 0.05$ vs MCAo alone. Scale bar A, B, D, E = 100 μm .

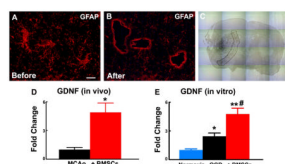


Fig 3.

A, B: GFAP immunofluorescent staining before and after cell capture; C: a LCM machine generated scanning picture after cell extraction by LCM shows that the collected GFAP positive reactive astrocytes are harvested from the ischemic boundary zone. D: GDNF gene expression in reactive astrocytes is significantly increased in BMSC treated animals compared to that of the MCAo alone rats. E: 5 hrs of OGD up-regulates GDNF gene expression of astrocytes, and coculture with BMSC during reoxygenation further augments this increase. * $P < 0.01$ vs. OGD; # $P < 0.05$ vs. normoxia. Scale bar = 25 μm .